



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/39	A1	(11) International Publication Number: WO 99/20300 (43) International Publication Date: 29 April 1999 (29.04.99)
<p>(21) International Application Number: PCT/US98/22405</p> <p>(22) International Filing Date: 22 October 1998 (22.10.98)</p> <p>(30) Priority Data: 60/062,617 22 October 1997 (22.10.97) US 60/062,716 22 October 1997 (22.10.97) US</p> <p>(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; 100 Church Street Southeast, Minneapolis, MN 55455 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): FIELDS, Gregg, B. [US/US]; 22708 Pickerel Circle, Boca Raton, FL 33428 (US). MCCARTHY, James, B. [US/US]; 2555 37th Avenue South, Minneapolis, MN 55406 (US).</p> <p>(74) Agent: MUETING, Ann, M.; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US).</p>	<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: INHIBITION OF TUMOR CELL ADHESION TO TYPE IV COLLAGEN</p> <p>(57) Abstract</p> <p>The invention provides polypeptides and peptide-conjugates and methods of their use. The polypeptide has an amino acid sequence which is a fragment of the continuous collagenous region of the major triple helical domain of the $\alpha 1$ chain of type IV collagen, wherein the polypeptide is in the all D-form. The peptide-conjugate includes a polypeptide fragment of the continuous collagenous region of the major triple helical domain of the $\alpha 1$ chain of type IV collagen covalently bonded to a non-peptide moiety.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

5 **INHIBITION OF TUMOR CELL ADHESION
 TO TYPE IV COLLAGEN**

Background of the Invention

 Type IV collagen is a distinctive glycoprotein which occurs
10 almost exclusively in basement membranes, structures which are found in the
 basal surface of many cell types, including vascular endothelial cells, epithelial
 cells, etc. Type IV collagen has a molecular weight (MW) of about 500,000 and
 consists commonly of two $\alpha 1$ (MW 185,000) chains and one $\alpha 2$ (MW 170,000)
 chain. Type IV collagen has two major proteolytic domains: a large, globular,
15 non-collagenous, NCI domain and another major triple-helical collagenous
 domain. The latter domain is interrupted by non-collagenous sequences of
 variable length. It is a complex and multidomain protein with different
 biological activities residing in different domains.

 Type IV collagen self-assembles to polymeric structures which
20 constitute the supportive frame of basement membranes. Various
 macromolecular components bind to type IV collagen, such as laminin,
 entactin/nidogen, and heparin sulfate proteoglycan. An additional function of
 type IV collagen is to mediate cell binding. A variety of cell types specifically
 adhere and spread onto type IV collagen-coated substrata. Various cell surface
25 proteins, a 47 kD protein, a 70 kD protein, and members of the superfamily of
 integrins have been reported to mediate cell binding to type IV collagen.

 Several synthetic peptides derived from the triple-helical region
 of type IV collagen are known to support cell adhesion and motility (G.B. Fields,
 Connect. Tissue Res., 31, 235-243 (1995)). A peptide incorporating $\alpha 1(IV)$
30 residues 1263-1277 and designated IV-H1 has been demonstrated to support
 melanoma cell adhesion (U.S. Patent No. 5,082,926 (Chelberg et al.); M.K.
 Chelberg et al., *J. Cell. Biol.*, 111, 261-270 (1990); K. Mayo et al., *Biochemistry*,
 30, 8251-8267 (1991); and C.G. Fields et al., *J. Biol. Chem.*, 268, 14153-14160
 (1993)). IV-H1 also supports melanoma cell motility and selectively inhibits cell
35 adhesion to type IV collagen (M.K. Chelberg et al., *J. Cell. Biol.*, 111, 261-270

5 (1990)). Melanoma cell motility is mediated by a chondroitin sulfate proteoglycan (D.J. Mickelson et al., *J. Cell. Biol.*, 115, 287a (1991)) and dependent upon IV-H1 conformation (M.K. Chelberg et al., *J. Cell. Biol.*, 111, 261-270 (1990); K. Mayo et al., *Biochemistry*, 30, 8251-8267 (1991)). However, these studies involved the all-L form of the polypeptide.

10 There is no general corollary that all-D forms of peptides will function in the same manner as all-L forms. D-amino acid substituted analogs of a Gly-Arg-Gly-Asp-Ser-Pro peptide have been studied for inhibition of rat kidney cell adhesion to either fibronectin (via the $\alpha_5\beta_1$ integrin) or vitronectin (via the $\alpha_v\beta_3$ integrin) (M.D. Pierschbacher et al., *J. Biol. Chem.*, 267, 14118-14121 (1992)). Substitution of Arg with D-Arg had no effect on the inhibitory
15 activities of the peptide, while substitution of Asp with D-Asp resulted in an inactive peptide. Thus, inhibition of integrin binding to either fibronectin or vitronectin by Arg-Gly-Asp sequences is sensitive to the peptide inhibitor stereochemistry. Additional studies which correlated the NMR-derived structures
20 of cyclic Arg-Gly-Asp analogs with inhibition of $\alpha_v\beta_3$ integrin binding to vitronectin indicated that the $\alpha_v\beta_3$ integrin interacts with both the Arg-Gly-Asp peptide side-chains and backbone (J. Wermuth et al., *J. Am. Chem. Soc.*, 119, 1328-1335 (1997)).

 In contrast, the laminin derived synthetic peptide LAM-L (A chain
25 residues 2097-2108) and its all D-enantiomer had near identical concentration-dependent activities for promotion of rat pheochromocytoma cell (PC12) attachment, inhibition of PC12 adhesion to laminin, and promotion of murine melanoma cell growth in mice (M. Nomizu et al., *J. Biol. Chem.*, 267, 14118-14121 (1992)). The cell surface receptor for LAM-L or LAM-D was not
30 identified. A synthetic combinatorial library has been used to select an all-D peptide (acetyl-Arg-Phe-Trp-Ile-Asn-Lys-NH₂) as a potent ligand for the μ opioid receptor (C.T. Dooley, *Science*, 266, 2019-2022 (1994)). The peptide was shown to be a full agonist, binding to the μ receptor and inducing a conformational change which allowed for signal transduction. In this case, the
35 all-L peptide was not active.

5 Schnolzer and Kent (M. Schnolzer et al., *Science*, 256, 221-225
(1992)) synthesized all-L and all-D HIV-1 proteases, then examined the chiral
specificity of the two enzymes using the substrate
2-aminobenzoyl-Thr-Ile-Nle-Nph-Gln-Arg-NH₂ (where Nph is
nitrophenylalanine). The synthetic all-L enzyme cleaved only the all-L, not the
10 all-D, version of 2-aminobenzoyl-Thr-Ile-Nle-Nph-Gln-Arg-NH₂, while the
synthetic all-D enzyme cleaved only the all-D substrate. The chiral specificity of
enzymes was established by these results.

The results of other enzyme studies are consistent with those from
the HIV-1 study, in that native (all-L) enzymes cleave only all-L substrates, not
15 all-D substrates. For example, trypsin cleaves all-L cecropin A but does not
cleave all-D cecropin A (D. Wade et al., *Proc. Natl. Acad. Sci. USA*, 87, 4761-
4765 (1990)). Further, trypsin cleaved L-Hep-III rapidly but did not hydrolyze
D-Hep-III (C. Li et al., *Biochemistry*, 36, 15404-15410 (1997)).

20 Summary of the Invention

The present invention provides polypeptides which represent an
all-D form of a fragment of the $\alpha 1$ chain of human type IV collagen derived from
the continuous collagenous region of the major triple helical domain. These
polypeptides can be prepared by conventional solid phase synthesis and
25 preferably include 15 amino acid residues. As used herein, an all-D polypeptide
may include amino acid residues that are not chiral and therefore are in neither
the D or the L form (e.g., glycine).

In one embodiment, the formula of the polypeptide is: gly-val-
lys-gly-asp-lys-gly-asn-pro-gly-trp-pro-gly-ala-pro. This specific polypeptide
30 formally substantially corresponds to isolated type IV collagen residues 1263-
1277 from the major triple helical region of the $\alpha 1$ chain of type IV collagen,
although all the amino acids are in the D-form where appropriate (gly is in
neither the L nor the D form). The single letter amino acid code for this
polypeptide is GVKGDKGNGPWPGAP. Herein, this specific polypeptide is
35 designated "D-IVH1".

5 The all-D polypeptide D-IV H1 was assayed for biological activity. It does not efficiently promote the adhesion and spreading of many cell types, and is not a potent attractant for melanoma cell motility. This is in contrast to the all-L form. However, like the all-L form, the all-D form efficiently inhibits tumor cell binding to type IV collagen, tumor cell invasion of
10 basement membranes, and tumor cell metastasis *in vivo*. Also, like the all-L form, the all-D form is highly specific in its cell binding properties. Therefore, it is believed that polypeptides such as D-IVH1 may be useful to (a) inhibit the metastasis and invasion of tumor cells, and (b) target cytotoxic agents to tumor cells. Since it is expected that further hydrolysis of the peptide D-IVH1 *in vitro*
15 or *in vivo* will yield some fragments of substantially equivalent bioactivity, such lower molecular weight peptides are also considered to be within the scope of the present invention.

 The present invention also provides peptide-conjugates wherein the all-D form, or the all-L form, of the polypeptides described herein,
20 particularly the IV-H1 peptide (e.g., a peptide incorporating $\alpha 1$ (IV) residues 1263-1277), is attached (covalently bonded) to a non-peptide moiety, such as a lipophilic C₁₀ alkyl "tail" and polyethylene glycol (PEG). Such conjugates inhibit tumor cell binding to type IV collagen.

 The polypeptides and peptide-conjugates described herein can
25 also include a cytotoxic agent for selective targeting of tumor cells for therapeutic effect. In such complexes, the cytotoxic agent is covalently bonded to a peptide portion, although it could be covalently bonded to a non-peptide moiety.

 The present invention also provides therapeutic methods. For
30 example, the present invention provides a method of inhibiting tumor cell binding (adhesion) to type IV collagen comprising contacting the tumor cell with a polypeptide or peptide-conjugate as described herein. Another method of the present invention involves inhibiting tumor cell invasion of a basement membrane. The method includes modulating the tumor cell with a polypeptide
35 or peptide-conjugate as described herein. The present invention also provides a

5 method of inhibiting tumor cell metastasis comprising modulating the tumor cell with a polypeptide or peptide-conjugate as described herein. Preferably, each of these methods is carried out *in vivo*. As used herein, "inhibiting" does not necessarily mean complete elimination of the activity, rather it means that the level of the activity (tumor cell binding, invasion, or metastasis) is decreased
10 relative to the level of that activity in the absence of the polypeptide or peptide-conjugate. The term "modulating" means bringing the polypeptide or peptide-conjugate in close proximity to, and preferably so close that it is in contact with, the tumor cell.

15 **Brief Description of the Drawings**

Figures 1A and 1B show the relative inhibition of M14#5 human melanoma cell adhesion to 10 µg/mL type IV collagen (TIV), fibronectin (FN), laminin (LM), or bovine serum albumin (BSA) by 100 µg/mL of L-IVH1, D-IVH1, or RI-IVH1 (a polypeptide having the sequence pro-ala-gly-pro-trp-gly-pro-asn-gly-lys-asp-gly-lys-val-gly, which is the all-D form synthesized in the reverse order and referred to as "Retro-Inverso"). Cells were preincubated with the peptides for 15 minutes and then added to the wells in the presence of the peptides for a 30-minute incubation period at 37°C. The data represent the means of triplicate points plus or minus the standard errors of the means.
20
25 Figures 1A and 1B represent different experiments run under the same conditions.

Figure 2A and B show the inhibition of M14#5 human melanoma cell invasion through MATRIGEL by 500 µg/mL (A) or 1 mg/mL (B) of L-IVH1, D-IVH1, or RI-IVH1 (a polypeptide having the sequence pro-ala-gly-pro-trp-gly-pro-asn-gly-lys-asp-gly-lys-val-gly, which is the all-D form synthesized in the reverse order and referred to as "Retro-Inverso"). Cells were mixed with the peptides and then tested for their ability to invade through MATRIGEL basement membrane (obtained from Collaborative Biomedical Products). The data represents the means of triplicate points plus or minus the standard errors of the means.
30
35

5 Figure 3 shows the inhibition of M14#5 human melanoma cell adhesion to 10 $\mu\text{g/mL}$ type IV collagen by D-IVH1(-Y) (closed squares), D-IVH1' (closed circles), D-IVH1(-Y)C10 (open squares), D-IVH1C'10 (open circles), or D-IVH1'PEG (starred circles). Cells were preincubated with the peptides for 15 minutes and then added to the wells in the presence of the
10 peptides for a 60-minute incubation period at 37°C. The data represent the means of triplicate points plus or minus the standard errors of the means.

 Figure 4 is a graph showing the inhibition of M14#5 human melanoma cell adhesion to 10 $\mu\text{g/mL}$ type IV collagen by D-IVH1' (closed squares), D-IVH1'C10 (open squares), or D-IVH1'PEG (closed circles). Cells
15 were preincubated with the peptides for 15 minutes and then added to the wells in the presence of the peptides for a 60-minute incubation period at 37°C. The data represent the means of triplicate points plus or minus the standard errors of the means.

20 Detailed Description of the Invention

 The structure of the two $\alpha 1$ chains and the single $\alpha 2$ chain of type IV collagen has been the subject of much study. The sequence of the $\alpha 1$ chain is shown in Figure 2 of U.S. Patent No. 5,082,926 (Chelberg et al.). The total number of amino acids per collagen molecule is approximately 4,550, with each
25 1(IV) chain containing approximately 1,390 amino acids.

 The inhibitory activities of IV-H1 synthesized with all-L amino acids (designation L-IVH1), all-D amino acids (designated D-IVH1), and IV-H1 synthesized in reverse sequence order with all-D amino acids (retro-inverso; designated RI-IVH1) were analyzed. The all-D IV-H1 inhibits melanoma cell
30 adhesion to type IV collagen (Figure 1) and invasion of MATRIGEL basement membrane (Figure 2) at least as well as does the all-L form. The retro-inverso form of IV-H1 has only weak inhibitory properties at best. Thus, the present invention provides polypeptides which represent an all-D form of a fragment of the $\alpha 1$ chain of human type IV collagen derived from continuous collagenous
35 region of the major triple helical domain.

5 Surprisingly, both the all-L and all-D versions of IV-H1 inhibit melanoma cell metastasis *in vivo* (Table 1). Also, the all-D version inhibits spontaneous Lewis lung tumor metastasis. These results are in contrast to that of Nomizu et al., *J. Biol. Chem.*, 267, 14118-14121 (1998), who found that an all-D laminin derived synthetic peptide LAM-L (A chain residues 2097-2108)
10 *increased* murine melanoma cell growth *in vivo* in comparison to no peptide.

The present invention also provides peptide-conjugates, i.e., where a non-peptide moiety is incorporated onto a polypeptide as described above, particularly onto the peptide IV-H1, for the all-D form as well as the all-L form of the polypeptide. Peptide-conjugates are typically created to improve the
15 bioavailability and subsequent half-life of peptide-based drugs *in vivo*. The peptide-conjugates of the present invention have been shown to inhibit adhesion of tumor cells to type-IV collagen, and are believed to provide inhibitory activity with respect to tumor cell invasion of basement membranes and tumor cell metastasis.

20 Preferably, the non-peptide moieties are typically those that impart some hydrophobic character to the peptide and are not readily hydrolyzed. Preferred non-peptide moieties include alkyl chains (typically, C₆-C₁₈ alkyls to provide, e.g., monoalkyl tails and dialkyl tails), phospholipids, and polyalkylene glycols. Specific examples include, for example, a lipophilic C₁₀ alkyl "tail" and
25 polyethylene glycol (PEG). Such conjugates can be synthesized by methods known in the art, particularly solid phase methods.

In certain specific embodiments, the non-peptide moiety can be any organic group having a long alkyl group (preferably, a linear chain). For example, the organic group can include at least two long alkyl groups
30 (preferably, linear chains) that are capable of forming lipid-like structures. This organic group also includes suitable functional groups for attachment to the peptide portion. Preferably, the organic group is attached to the peptide portion through a linker group having suitable functionality such as ester groups, amide groups, and combinations thereof. Suitable non-peptide moieties can be derived
35 from compounds such as, for example, alkylamines, alkylesters, and

5 phospholipids.

When lipophilic non-peptide moieties are used, bilayer membrane systems can be formed, where the lipid moiety serves as an anchor for the functional head group to the lipid assembly. For example, such peptide-conjugates may form a great variety of structures in solution including micelles and vesicles. They can also be mixed with vesicle-forming lipids, such as dilauryl phosphatidylcholine, to form stable mixed vesicles with peptide head groups. These can be used as delivery vesicles for the peptide and optionally a cytotoxic agent. For example, a drug targeting system against melanoma cells can be designed using such complexes.

15 In the examples discussed below, non-peptide moieties were added to one of two forms of all-D IV-H1; one containing just the IV-H1 sequence [designated D-IVH1(-Y)], and one containing the IV-H1 sequence and a C-terminal Tyr residue (designated D-IVH1'). C₁₀-D-IV-H1 [designated either D-IVH1(-Y)C10 or D-IVH1'C10] and PEG₁₉₀₀-D-IV-H1 (designated D-IVH1'PEG) were tested for inhibition of M14 human melanoma cell adhesion to type IV collagen. Both C₁₀-D-IV-H1 and PEG₁₉₀₀-D-IV-H1 inhibited melanoma cell adhesion to type IV collagen in a dose-dependent fashion (Figure 3). The IV-H1 sequence and the IV-H1 containing a C-terminal Tyr residue were tested. There was more effective inhibition when the Tyr was not present (Figure 3).

25 C₁₀-D-IV-H1 [designated D-IVH1'C10] and PEG₁₉₀₀-D-IV-H1 (designated D-IVH1'PEG) were subsequently retested for inhibition of M14 human melanoma cell adhesion to type IV collagen. D-IV-H1, C₁₀-D-IV-H1, and PEG₁₉₀₀-D-IV-H1 all inhibited melanoma cell adhesion to type IV collagen in similar dose-dependent fashions (Figure 4). Thus, adding a conjugate to the D-IV-H1 sequence does not compromise the inhibitory properties of D-IV-H1, and may improve the *in vivo* half-life of this potential therapeutic.

The present invention also provides complexes and methods wherein a cytotoxic agent can be delivered to a cell. That is, the polypeptides or peptide-conjugates described herein can be used to target specific tumor cells, bind thereto, optionally invade the cellular structure, and deliver a cytotoxic

35

5 agent. Examples of cytotoxic agents include DNA intercalators, metal chelators, alkylating agents, and membrane disrupting agents. Examples of specific such agents include risin A, dioxorubicin, and mitomycin C.

The complexes (polypeptides and conjugates with or without cytotoxic agents attached thereto) of the present invention can be made by a
10 variety of solid-phase or solution techniques. Although the polypeptides can be prepared by other methods (e.g., solution methods) and then attached to a support material for subsequent coupling with a non-peptide moiety, it is preferred that standard solid-phase organic synthesis techniques, such as solid-phase peptide synthesis (SPPS) techniques be used for preparation of the
15 peptides as well as the conjugates.

Preferably, solid-phase peptide synthesis involves a covalent attachment step (i.e., anchoring) that links the nascent peptide chain to a support material (typically, an insoluble polymeric support) containing appropriate functional groups for attachment. Subsequently, the anchored peptide is
20 extended by a series of addition (deprotection/coupling) cycles that involve adding N^α-protected and side-chain-protected amino acids stepwise in the C to N direction. Once chain assembly has been accomplished, protecting groups are removed and the peptide is cleaved from the support. Typically, the non-peptide moiety and/or the cytotoxic agent is added to the peptide before the protecting
25 groups are removed.

Typically, SPPS begins by using a handle to attach the initial amino acid residue to a functionalized support material. A handle (i.e., linker) is a bifunctional spacer that, on one end, incorporates features of a smoothly cleavable protecting group, and on the other end, a functional group, often a
30 carboxyl group, that can be activated to allow coupling to the functionalized support material. Known handles include acid-labile p-alkoxybenzyl (PAB) handles, photolabile o-nitrobenzyl ester handles, and handles such as those described by Albericio et al., *J. Org. Chem.*, 55, 3730-3743 (1990) and references cited therein, and in U.S. Patent Nos. 5,117,009 (Barany) and
35 5,196,566 (Barany et al.).

5 For example, if the support material is prepared with amino-functional monomers, typically, the appropriate handles are coupled quantitatively in a single step onto the amino-functionalized supports to provide a general starting point of well-defined structures for peptide chain assembly. The handle protecting group is removed and the C-terminal residue of the N^α-
10 protected first amino acid is coupled quantitatively to the handle. Once the handle is coupled to the support material and the initial amino acid or peptide is attached to the handle, the general synthesis cycle proceeds. The synthesis cycle generally consists of deprotection of the N^α-amino group of the amino acid or peptide on the support material, washing, and, if necessary, a neutralization step,
15 followed by reaction with a carboxyl-activated form of the next N^α-protected amino acid. The cycle is repeated to form the peptide of interest. Solid-phase peptide synthesis methods using functionalized insoluble support materials are well known. See, for example, Merrifield, *J. Am. Chem. Soc.*, 85, 2149 (1963); Barany and Merrifield, In *Peptides*, Vol. 2, pp. 1-284 (1979); Barany et al., *Int. J. Peptide Protein Res.*, 30, 705-739 (1987); Fields et al., In *Synthetic Peptides: A User's Guide* (G.A. Grant, Ed.), Chapter 3, pp. 77-183, W.H. Freeman and Co., NY (1992); and Fields et al., *Int. J. Peptide Protein Res.*, 35, 161-214 (1990).

 When SPPS techniques are used to synthesize the polypeptides described herein on the support material, Fmoc methodologies are preferably
25 used. This involves the use of mild orthogonal techniques using the base-labile N^α-9-fluorenylmethyloxycarbonyl (Fmoc) protecting group. Fmoc amino acids can be prepared using fluorenylmethyl succinimidyl carbonate (Fmoc-OSu), Fmoc chloride, or [4-(9-fluorenylmethyloxycarbonyloxy)phenyl]dimethylsulfonium methyl sulfate
30 (Fmoc-ODSP). The Fmoc group can be removed using piperidine in dimethylformamide (DMF) or N-methylpyrrolidone, or using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF. After Fmoc removal, the liberated N^α-amine of the supported resin is free and ready for immediate attachment of the non-peptide moiety without an intervening neutralization step.
35 The immobilized conjugate can then be removed, for example, using

- 5 trifluoroacetic acid (TFA) at room temperature. Such Fmoc solid-phase peptide synthesis methodologies are well known to one of skill in the art and are discussed in Fields et al., In *Synthetic Peptides: A User's Guide* (G.A. Grant, Ed.), Chapter 3, pp. 77-183, W.H. Freeman and Co., NY (1992); and Fields et al., *Int. J. Peptide Protein Res.*, 35, 161-214 (1990).
- 10 A variety of support materials for preparation of the complexes of the present invention can be used. They can be of inorganic or organic materials and can be in a variety of forms (e.g., membranes, particles, spherical beads, fibers, gels, glasses, etc.). Examples include, porous glass, silica, polystyrene, polyethylene terephthalate, polydimethylacrylamides, cotton, paper, and the like.
- 15 Examples of suitable support materials are described by Fields et al., *Int. J. Peptide Protein Res.*, 35, 161-214 (1990); and *Synthetic Peptides: A User's Guide* (G.A. Grant, Ed.), Chapter 3, pp. 77-183, W.H. Freeman and Co., NY (1992). Functionalized polystyrene, such as amino-functionalized polystyrene, aminomethyl polystyrene, aminoacyl polystyrene, p-methylbenzhydrylamine
- 20 polystyrene, or polyethylene glycol-polystyrene resins can be used for this purpose.

Objects and advantages of this invention are further illustrated by the following examples, but the particular materials and amounts thereof recited in these examples, as well as other conditions and details, should not be construed to

25 unduly limit this invention.

Synthesis of the Polypeptide

- Methods for the synthesis of peptides have been described extensively previously (C. Fields, et al., *J. Biol. Chem.*, 268, 14153-14160
- 30 (1993); A. Miles et al., *J. Biol. Chem.*, 269, 30939-30945 (1994); Y.-C. Yu et al., *J. Am. Chem. Soc.*, 118, 12515-12520 (1996); G. Fields et al., *Synthetic Peptides: A User's Guide*, (Grant, G.A., ed.), pp. 77-183, W. H. Freeman & Co., New York (1992); C. Fields et al., *Biopolymers*, 33, 1695-1707 (1993); C. Fields et al., *Peptide Res.*, 6, 39-47 (1993); G. Rao et al., *J. Biol. Chem.*, 269,
- 35 13899-13903 (1994); H. Nagase et al., *J. Biol. Chem.*, 269, 20952-20957 (1994);

- 5 J. Lauer et al., *Lett. Peptide Sci.*, 1, 197-205 (1995); B. Grab et al., *J. Biol. Chem.*, 271, 12234-12240 (1996); J. Lauer et al., *J. Med. Chem.*, 40, 3077-3084 (1997); C. Fields et al., *Anal. Biochem.*, 231, 57-64 (1995)). These synthetic methods involved solid-phase techniques using Fmoc-amino acids on an ABI 431A peptide synthesizer. For the preparation of peptide-conjugates, either
10 decanoic acid [$\text{CH}_3-(\text{CH}_2)_8-\text{CO}_2\text{H}$, designated C_{10}], or PEG of MW 1900 Da was coupled to the resin-bound peptide using $N-[(1H\text{-benzotriazol-1-yl})(\text{dimethylamino})\text{methylene}]-N\text{-methylmethanaminium hexafluoro-phosphate } N\text{-oxide (HBTU)}$ as described previously (Y.-C. Yu et al., *J. Am. Chem. Soc.*, 120, in press).
- 15 Peptides and peptide-conjugates were purified using reversed-phase high performance liquid chromatography (RP-HPLC) on a Rainin AutoPrep System. Peptides were purified with a Vydec 218TP152022 C_{18} column (15-20 μm particle size, 300 Angstrom pore size, 250 x 25 mm) at a flow rate of 5.0 ml/minute. The elution gradient was either 0-60% B or 0-100%
20 B in 60 minutes, where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile. Detection was at 229 nm. Peptide-conjugate purification was achieved using either the method described above or a Vydac 214TP152022 C_4 column (15-20 μm particle size, 300 Angstrom pore size, 250 x 22 mm) at a flow rate of 10 ml/minute. The elution gradient was 55-90% B in 20 minutes,
25 where A was 0.05% TFA in water and B was 0.05% TFA in acetonitrile. Detection was at 229 nm. Analytical RP-HPLC was performed on a Hewlett-Packard 1090 Liquid Chromatograph equipped with a Hypersil C_{18} column (5 μm particle size, 120 Angstrom pore size, 200 x 2.1 mm) at a flow rate of 0.3 ml/minute. The elution gradient was 0-60% B in 45 minutes, where A and B
30 were the same as for peptide purification. Diode array detection was at 220, 254, and 280 nm.

Purity and composition of the final compounds was assured by Edman degradation sequence analysis of the peptides and analytical RP-HPLC and laser desorption mass spectrometry (LDMS) of the peptides and
35 peptide-conjugates. Edman degradation sequence analysis was performed on an

5 Applied Biosystems 477A Protein Sequencer/120A Analyzer. LDMS was performed on a Hewlett Packard matrix-assisted laser desorption time-of-flight mass spectrometer.

To synthesize either a peptide or peptide-conjugate containing a cytotoxic agent, one would need to assemble the toxin, such as the risin A chain,
10 onto the α -amino group of the peptide and the α - or ϵ -amino group of the peptide-conjugate. For example, the all-D IV-H1 is synthesized, and the risin A chain sequence (Gln-Tyr-Ile-Lys-Ala-Asn-Ser-Lys-Phe-Ile-Gly-Ile-Thr-Glu) is assembled onto the *N*-terminus of the resin-bound IV-H1 sequence by standard solid-phase methods (G. Fields et al., *Synthetic Peptides: A User's Guide*
15 (Grant, G.A. ed.), pp. 77-183, W.H. Freeman & Co., New York (1992)). A spacer such as 6-aminohexanoic acid may or may not be included between the IV-H1 and risin A sequences. Alternatively, for peptide-conjugates, the all-D IV-H1 is synthesized, an Fmoc-Lys(Dde) residue is incorporated (where Dde is 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-ethyl), the Fmoc group is
20 removed, and the risin A chain sequence is added to the resin-bound peptide. The Dde group is removed with hydrazine (C. Fields et al., *Biopolymers*, 33, 1695-1707 (1993) and the conjugate (alkyl tail or PEG) is added to the *N*- ϵ -amino group of the resin-bound peptide. The peptide or peptide-conjugate is then purified and characterized as described above.

25

Cell Culture

Human melanoma cells were cultured in Eagle's minimum essential media supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate, 0.1 mg/mL gentamicin (Boehringer Mannheim, Indianapolis, IN), 50
30 units/mL penicillin, and 0.05 mg/mL streptomycin. Cells were passaged 8 times and then replaced from frozen stocks of early passage cells to minimize phenotypic drift. All cells were maintained at 37°C in a humidified incubator containing 5% CO₂. All media reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

35

5

Labeling of Peptides

Assays are first performed to quantitate the amount of each peptide adsorbed to the wells after adsorption and rinsing. Synthetic peptides are radiolabeled by reductive methylation using sodium cyanoborohydride and 3H-formaldehyde. By this technique, the ϵ -amino groups of Lys and the α -amino terminus become labeled. The radiolabeled substrate is added to microtiter wells and incubated overnight. Wells are blocked, then rinsed. Lysis buffer (0.5 M NaOH, 1% SDS) is then used to remove the radioactivity for quantitation.

Adhesion Assay

15

Adhesion of cells was determined as described previously (C. Fields et al., *J. Biol. Chem.*, 268, 14153-14160 (1993); A. Miles et al., *J. Biol. Chem.*, 269, 30939-30945 (1994); C. Li et al., *Biochemistry*, 36, 15404-15410 (1997); J. Lauer et al., *J. Med. Chem.*, 40, 3077-3084 (1997)). Briefly, peptides were dissolved in 1 mL of water or DMSO-water (1:9), diluted to desired concentrations with PBS, and adsorbed directly onto 96-well polystyrene Immulon 1 plates (Dynatech Laboratories Inc., Chantilly, VA) overnight at 37°C. Nonspecific binding sites were blocked with 2 mg/mL ovalbumin in phosphate buffered saline (PBS) for 2 hours at 37°C. Cells were radiolabeled overnight with 20 μ Ci/mL Tran 35 S-Label™ (>1000 Ci/mmol specific activity; ICN, Costa Mesa, CA). Cells were released from tissue culture flasks with 37°C PBS containing 0.05% trypsin and 0.53 mM EDTA, then washed several times with PBS. Cells were added to the wells at a density of 50,000 cells/mL in a total volume of 100 μ L of the respective cell media containing 2 mg/mL ovalbumin and incubated for 2 hours at 37°C. Wells were washed several times with PBS containing 2 mg/mL ovalbumin and the remaining adherent cells were lysed and radioactivity determined as described (C. Fields et al., *J. Biol. Chem.*, 268, 14153-14160 (1993); A. Miles et al., *J. Biol. Chem.*, 269, 30939-30945 (1994); C. Li et al., *Biochemistry*, 36, 15404-15410 (1997); J. Lauer et al., *J. Med. Chem.*, 40, 3077-3084 (1997)). Adhesion percentages were based on total counts of radioactivity added to each well.

Competition of cell adhesion assays were performed as described previously (A. Miles et al., *J. Biol. Chem.*, 269, 30939-30945 (1994); C. Li et al., *Biochemistry*, 36, 15404-15410 (1997)) using substrate at concentrations which provide $\geq 50\%$ initial cell adhesion. Cells were preincubated for 30 minutes at 37°C with various concentrations of the inhibitory peptide, then the cells, in the continued presence of the inhibitor, are added to the wells and allowed to adhere for 30 minutes at 37°C.

The invention will be further described by reference to the following detailed example.

15 **Example 1**

L-IVH1, D-IVH1, and RI-IVH1 were tested for their ability to inhibit metastasis *in vivo* as described previously (I. Saiki et al., *Jpn. J. Cancer Res.*, 84, 326-335 (1993)). Highly metastatic A375SM human melanoma cells (A375SM melanoma cells supplied by Dr. James B. McCarthy, University of Minnesota, who had originally obtained them from Dr. I.J. Fidler, M.D. Anderson Hospital, Houston, TX) were pre-incubated several different concentrations of L-IVH1, D-IVH1, and RI-IVH1 (Table 1). The cells were then injected into the lateral tail veins of immunocompromised (KSN nude female) mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), which had 24 hours prior to this been injected with 20 μ L of anti-asialo GM1 antisera (Shizuoda Laboratory Animal Center, Hamamatsu, Japan). After 50 days, the mice were sacrificed and the number of lung metastatic nodules was quantified in a blinded fashion. The data represent the means of 5 animals/group, plus or minus the standard deviations (SD) of the means. The all-L and all-D versions of IV-H1 were found to inhibit melanoma cell metastasis *in vivo* (Table 1). It was also found that a dose of 100 μ g/mouse of D-IVH1, initiated one day after tumor implantation, would inhibit spontaneous Lewis lung tumor metastasis by 50%. These results are in contrast to that of Nomizu et al., *J. Biol. Chem.*, 267, 14118-14121 (1998), who found that an all-D laminin derived synthetic peptide LAM-L (A chain residues 2097-2108) increased murine melanoma cell growth *in vivo*.

5

Table 1: Effects of IV-H1 peptide variants on experimental lung metastasis produced by intravenous injection of human A375SM melanoma cells.

10	Peptide	Dose	Lung metastases on day 50
		($\mu\text{g}/\text{mouse}$)	<u>mean + SD (range)</u>
	Control (PBS)	0	90 + 15 (80-117)
	L-IVH1	10	93 + 11 (81-107)
	L-IVH1	100	50 + 12 (36-62)
15	L-IVH1	1000	16 + 13 (4-34)
	D-IVH1	10	88 + 12 (65-96)
	D-IVH1	100	43 + 10 (31-54)
	D-IVH1	1000	31 + 10 (21-46)
	RI-IVH1	10	86 + 12 (72-102)
20	RI-IVH1	100	84 + 8 (76-96)
	<u>IR-IVH1</u>	<u>1000</u>	<u>64 + 9 (54-77)</u>

The inhibitory behaviors of D-IVH1 have also been examined by synthesizing several peptide-conjugates, i.e., where a non-peptide moiety is incorporated onto IV-H1. Peptide-conjugates are created to improve the bioavailability and subsequent half-life of peptide-based drugs *in vivo*. Two conjugates have been studied: a lipophilic C₁₀ alkyl "tail" and polyethylene glycol (PEG). Conjugates were added to one of two forms of all-D IV-H1; one containing just the IV-H1 sequence [designated D-IVH1(-Y)], and one containing the IV-H1 sequence and a C-terminal Tyr residue (designated D-IVH1'). The C₁₀ alkyl tail was coupled to resin-bound all-D IV-H1 and the product purified and characterized using methods described previously (P. Berndt et al., *J. Am. Chem. Soc.*, 117, 95159-9522 (1995); and Y.C. Yu, *J. Am. Chem. Soc.*, 118, 12515-12520 (1996)). PEG of MW 1900 was coupled to resin-bound all-D IV-H1 and the product purified and characterized as described

5 previously (P. Berndt et al., *J. Am. Chem. Soc.*, 117, 9515-9522 (1995); Y.C. Yu et al., *J. Am. Chem. Soc.*, 118, 12515-12520 (1996); Y.A. Lu et al., *Peptide Res.*, 6, 140-146 (1993); and Y.C. Yu et al., *J. Am. Chem. Soc.*, 120, 9979-9987 (1998)). C₁₀-D-IV-H1 [designated either D-IVH1(-Y)C10 or D-IVH1'C10] and PEG₁₉₀₀-D-IV-H1 (designated D-IVH1'PEG) were tested for inhibition of M14
10 human melanoma cell adhesion to type IV collagen using an assay previously described (A.J. Miles et al., *J. Biol. Chem.*, 269, 30939-30945 (1994)). Both C₁₀-D-IV-H1 and PEG₁₉₀₀-D-IV-H1 inhibited melanoma cell adhesion to type IV collagen in a dose-dependent fashion (Figure 3). The IV-H1 sequence and the IV-H1 containing a C-terminal Tyr residue were tested. There was more
15 effective inhibition when the Tyr was not present (Figure 3).

C₁₀-D-IV-H1 [designated D-IVH1'C10] and PEG₁₉₀₀-D-IV-H1 (designated D-IVH1'PEG) were subsequently retested for inhibition of M14 human melanoma cell adhesion to type IV collagen. D-IV-H1, C₁₀-D-IV-H1, and PEG₁₉₀₀-D-IV-H1 all inhibited melanoma cell adhesion to type IV collagen in
20 similar dose-dependent fashions (Figure 4). Thus, adding a conjugate to the D-IV-H1 sequence does not compromise the inhibitory properties of D-IV-H1, and may improve the *in vivo* half-life of this potential therapeutic.

The complete disclosures of the patents, patent documents, and
25 publications cited herein are incorporated by reference in their entirety as if each were individually incorporated. Various modifications and alterations to this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention. It should be understood that this invention is not intended to be unduly limited by the illustrative embodiments and
30 examples set forth herein and that such examples and embodiments are presented by way of example only with the scope of the invention intended to be limited only by the claims set forth herein as follows.

5 **WHAT IS CLAIMED IS:**

1. A polypeptide having an amino acid sequence which is a fragment of the continuous collagenous region of the major triple helical domain of the $\alpha 1$ chain of type IV collagen, wherein the polypeptide is in the all D-form.
10
2. The polypeptide of claim 1 wherein the amino acid sequence corresponds substantially to amino acid residues 1263 through 1277 of the continuous collagenous region of the major triple helical domain of the $\alpha 1$ chain of type IV collagen.
15
3. The polypeptide of claim 2 having 15 amino acid residues in the D-form where appropriate.
- 20 4. The polypeptide of claim 3 having the sequence gly-val-lys-gly-asn-pro-gly-trp-pro-gly-ala-pro.
5. The polypeptide of claim 1 further comprising a cytotoxic agent covalently bonded thereto.
25
6. The polypeptide of claim 1 which inhibits binding of tumor cells to type IV collagen.
7. The polypeptide of claim 1 which inhibits tumor cell invasion into basement membranes.
30
8. The polypeptide of claim 1 which inhibits tumor cell metastasis.
9. A peptide-conjugate comprising a polypeptide fragment of the continuous collagenous region of the major triple helical domain of the
35

- 5 α 1 chain of type IV collagen covalently bonded to a non-peptide moiety.
10. The peptide-conjugate of claim 9 wherein the polypeptide fragment is in the all D-form.
- 10 11. The peptide-conjugate of claim 9 wherein the polypeptide fragment is in the all L-form.
12. The peptide-conjugate of claim 9 wherein the amino acid sequence of the polypeptide fragment corresponds substantially to amino acid residues
- 15 1263 through 1277 of the continuous collagenous region of the major triple helical domain of the α 1 chain of type IV collagen.
13. The peptide-conjugate of claim 12 having 15 amino acid residues in the D-form where appropriate.
- 20 14. The peptide-conjugate of claim 13 having the sequence gly-val-lys-gly-asp-lys-gly-asn-pro-gly-trp-pro-gly-ala-pro.
15. The peptide-conjugate of claim 9 further comprising a cytotoxic agent
- 25 covalently bonded thereto.
16. A method of inhibiting tumor cell binding to type IV collagen comprising contacting the tumor cell with a polypeptide of claim 1 or a peptide-conjugate of claim 9.
- 30 17. A method of inhibiting tumor cell invasion of a basement membrane comprising modulating the tumor cell with a polypeptide of claim 1 or a peptide-conjugate of claim 9.
- 35 18. A method of inhibiting tumor cell metastasis comprising modulating the

5 tumor cell with a polypeptide of claim 1 or a peptide-conjugate of claim
9.

19. The method of any of claims 16-18 which is carried out *in vivo*.

Figure 1A

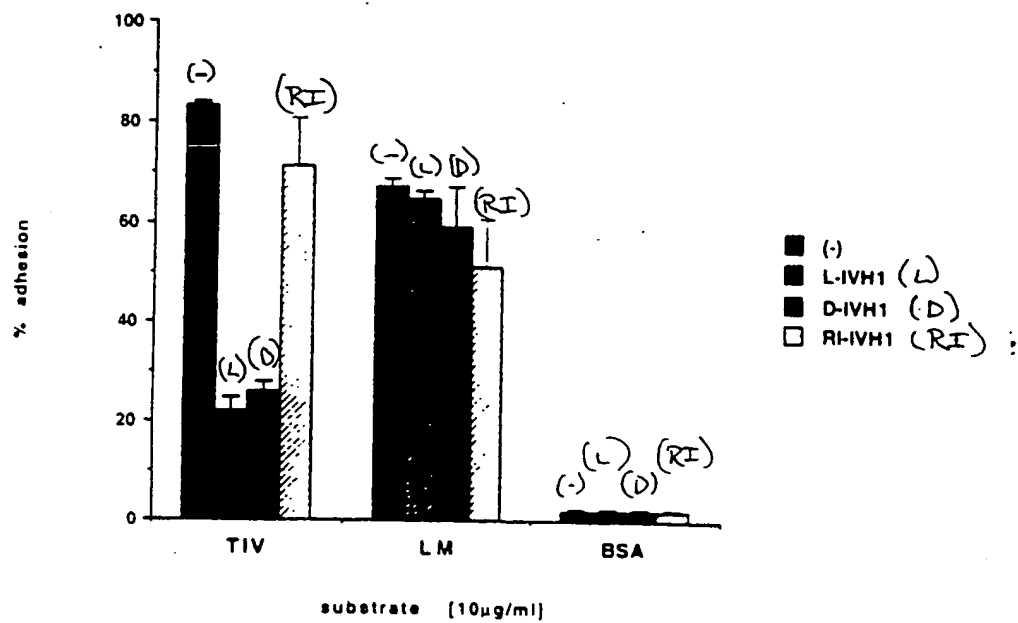


Figure 1B

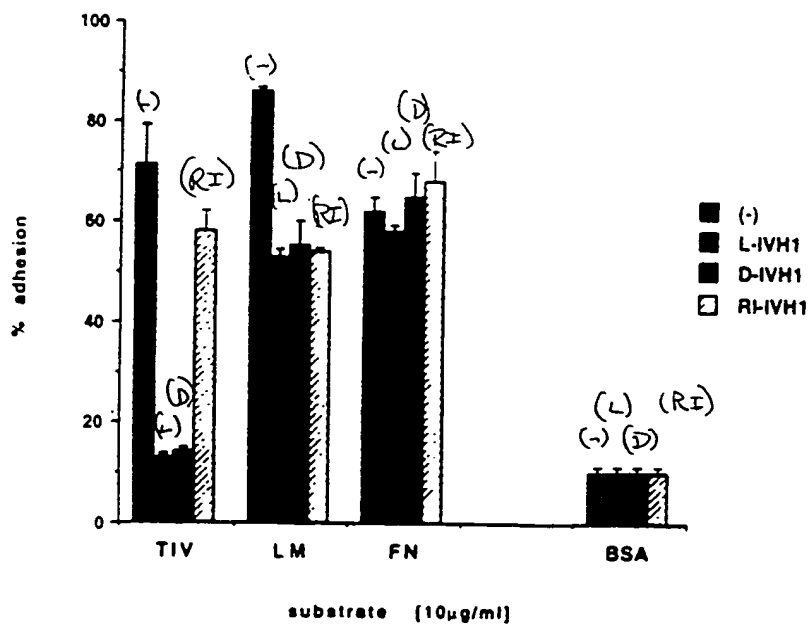


Figure 2A

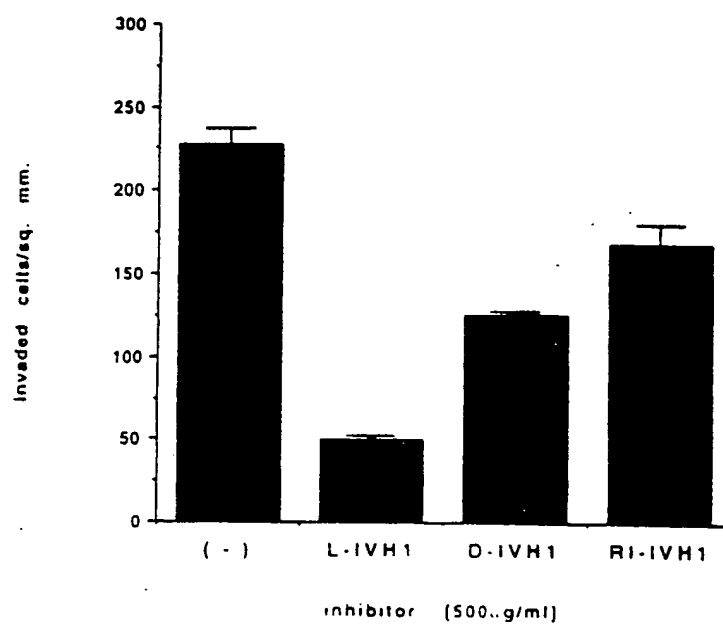
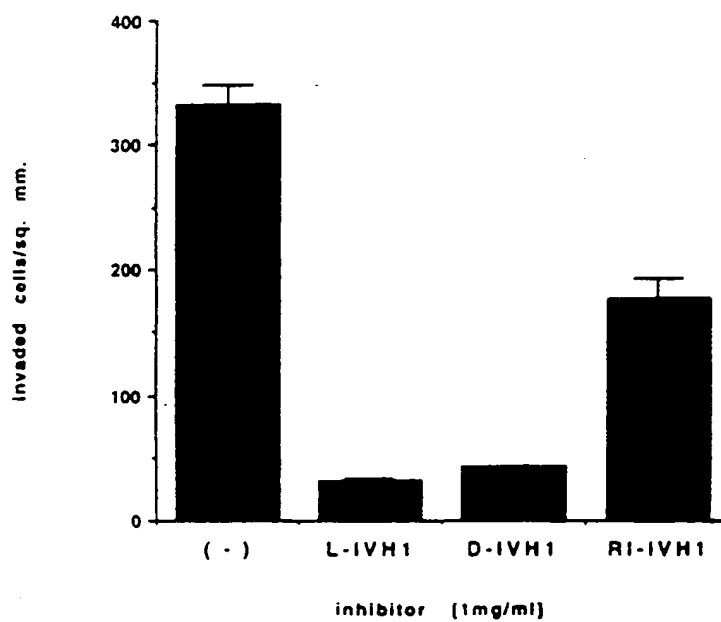
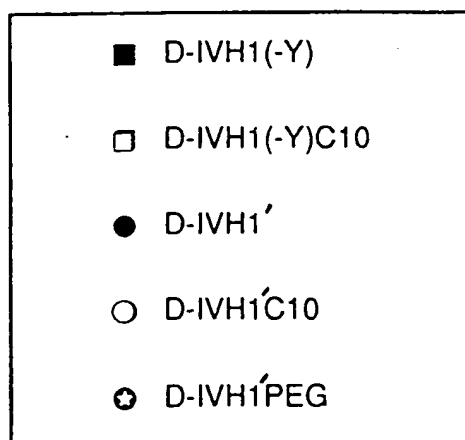
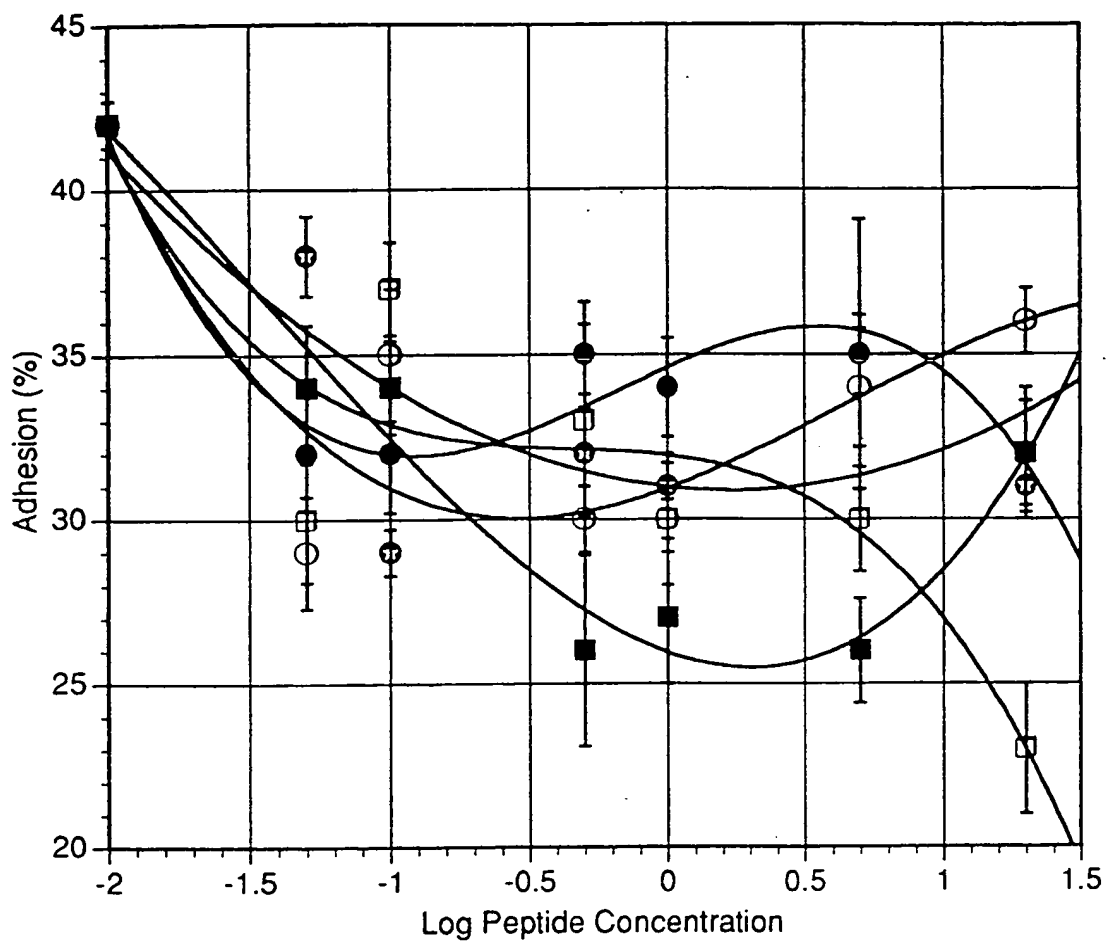


Figure 2B





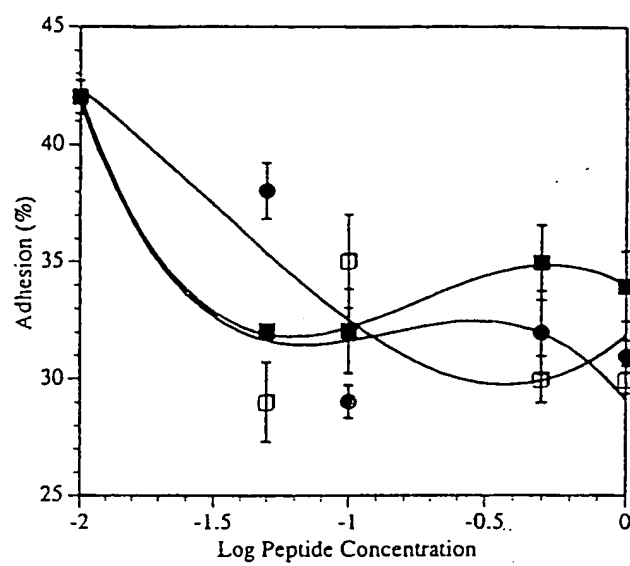


Figure 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/22405

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	CHANGFEN LI ET AL.: "AN ALL-D AMINO ACID PEPTIDE MODEL OF ALPHA1(IV)531-543 FROM TYPE IV COLLAGEN BINDS THE ALPHA3BETA1 INTEGRIN AND MEDIATES TUMOR CELL ADHESION, SPREADING, AND MOTILITY" BIOCHEMISTRY., vol. 36, no. 49, 9 December 1997, pages 15404-15410, XP002095057 EASTON, PA US cited in the application see the whole document ---	1,7,8, 16-19
Y,P	WO 98 08098 A (FLEDELIUS CHRISTIAN ;QVIST PER (DK); CLOOS PAUL (DK); OSTEOMETER B) 26 February 1998 see page 11, line 25 - line 32 --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

1 March 1999

Date of mailing of the international search report

16/03/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 98/22405

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ²	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 91 08755 A (UNIV MINNESOTA) 27 June 1991 cited in the application see page 5, line 35 - page 8, line 25 -----</p>	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 22405

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 16-19
because they relate to subject matter not required to be searched by this Authority, namely:
See FURTHER INFORMATION sheet PCT/ISA/210
2. ☒ Claims Nos.: 16-19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 98 22405

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 16 to 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Claims Nos.: 16-19

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/22405

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9808098 A	26-02-1998	AU 3943597 A	06-03-1998
WO 9108755 A	27-06-1991	US 5082926 A	21-01-1992
		AT 163859 T	15-03-1998
		CA 2071512 A	15-06-1991
		DE 69032136 D	16-04-1998
		DE 69032136 T	03-12-1998
		EP 0505488 A	30-09-1992
		JP 5503097 T	27-05-1993